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Restriction Fragment Length Polymorphism of DRB3 Exon 2 of BoLA Class II Gene Complex in Relation to Phenotypic Traits of Holstein Dairy Cows

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Abstract

IN this study, PCR-RFLP was performed to detect the potential association among the genotypic polymorphism of BoLA-DRB3 exon 2 in 20 Holstein dairy cows and recurrent cystic ovary, mastitis, milk production and foot and mouth disease virus infection. Based on the combination between the RFLP patterns produced by two restriction enzymes, 5 patterns were seen. The profile AB showed the highest incidence of recurrent cystic ovary, as four animals needed mating for three times for conception. Concerning the milk yield, a cow with the yield (BB profile showed the highest 5200 Kg/305 days) followed by those with the BC profile. Cows with the RFLP profiles AA, AB and BB did not attract FMD infection during the endemics that attacked the flock. Meanwhile, two cows with the BC profiles showed severe FMD symptoms. Animals with the rest of profiles showed mild to moderate FMD symptoms according to the farm records. It is noticed that cows with profiles AA, AB and BB were affected with clinical mastitis 3 to 4 times through the production period (305 days). Cows with the profile BC were the least affected with subclinical mastitis. In conclusion, polymorphism of BoLA-DRB3 exon 2 in the studied Holstein cows showed sorts of association with productive and immunological traits and can be a reliable genetic marker for selection of animals with better traits. However, more investigation is needed on a bigger number of animals and more traits.

Key words: BoLA DRB3, RFLP, PAGE, Holstein cows, phenotypic traits.

Introduction

Cattle productive and reproductive performance strategies has been recently supported by selection assisted by the use of molecular markers [1]. Some of those markers are related to the immune responses modulation in addition to all production and reproduction aspects of an animal as well. BoLA DRB3.2, an exon of the major histocompatibility gene complex with more than 100 polymorphic sites, is one of the favourable gene markers [2]. This locus has been broadly related to certain immunological traits [3, 4], resistance or susceptibility to various diseases [5], and production traits and milk quality [6,7,8].

In 1978, Spooner and colleagues announced, for the first time the term BoLA denoting the bovine leukocyte antigen in both *Bos taurus* and *Bos indicus* cattle species. This was during the First International Bovine Leukocyte Antigen workshop. The BoLA complex is located on the short arm of chromosome 23 and is organized in three classes; I, II and III [9]

BoLA genes are located in two regions of chromosome 23 of cattle. BoLA class II sequence was a preferred genetic marker as it has been found highly polymorphic and it required simple DNA analysis based on polymerase chain reaction in addition to its role in immunity [10,11].

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DR and *DQ* genes represent the *BoLA* class IIa sub-region: DRA, DRB1, DRB2, DRB3, DQA1, DQA2, DQB1, and DQB2. DRB genes are represented by three loci: DRB1, DRB2 and DRB3. While DRB1 is a pseudogene and DRB2 is transcribed at a low level, DRB3 is highly polymorphic as 103 alleles has been already among the best defined loci in cattle. The BoLA-DRB3 gene has been extensively studied for its extensive polymorphism potential associations with disease resistance or susceptibility [12].

The exon 2 of BoLA-DRB3, the most variable region of the class II alleles, encodes peptide-binding residues [13, 14]. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) has been a widely method applied to study polymorphism of the exon 2 of BoLA-DRB3 is PCR-RLFP. The method was developed by [15] which consisted of a hemi-nested PCR amplification followed by restriction enzyme digestion of the 284 bp amplicon of exon 2 of the DRB3 gene. Restriction digestion was carried out with the restriction enzymes RsaI, HaeIII and BstYI in separate reactions followed by polyacrylamide gel electrophoresis (PAGE).

Associations between *BoLA* alleles and diseases and some phenotypic traits has bee elucidated in cattle. It was documented that the BoLA-DRB3.2 alleles potentially affect many traits related to immunity, mastitis incidence [16,17] BoLA-DRB3.2 alleles were found to be associated with resistance to bovine leukosis virus persistent leukemia as well as various forms of mastitis in Mongolian and Kalmyk cattle breeds. Both breeds were characterized by a wide diversity of BoLA-DRB3 gene alleles and genotypes [18].

In a study conducted in Iran, certain Holstein cattle BoLA-DRB3.2 alleles were associated with a lower risk of cystic ovarian disease as well as the resistance to mastitis and bovine leukemia virus infection. Likewise, Ayrshire and Black Pied cattle breeds of Russian selection showed BoLA-DRB3 allelic association with resistance or susceptibility to persistent leukemia due to bovine leukemia virus infection [19,20].

A 58.7% prevalence of BoHV-1 infection in a population of Holstein cattle using indirect ELISA. A significant association of the susceptibility to infection with the allele 37 of the BoLA DRB3 exon 2. Meanwhile, a significant association of BoLA DRB3.2 gene with milk production and fat yield in primiparous (alleles 11 and 28) as well as services per conception (alleles 22 and 28) in multiparous [21].

Accordingly, the aim of this work was to figure out the possible association of BoLA DRB3.2 polymorphisms with productive and reproductive traits of a Holstein dairy cows' population in south-west of Cairo, Egypt. Also, resistance to or susceptibility for FMD virus infection and mastitis was investigated. This was to establish allelic preferences or exclusion towards the investigated phenotypes.

Material and Methods

Blood Samples

Whole blood samples were collected by venipuncture on EDTA-Na_2 treated tubes and transported while cold to the laboratory with minimum delay.

DNA Extraction

DNA was extracted employing the Genomic DNA Isolation kit (GeneDirex Inc., Taiwan). Steps described by the manufacturer were strictly followed. Briefly, 300 µl of blood was transferred to a 1.5 ml microfuge tube and 900 µl of the CR buffer was mixed with the blood followed by a 10 minute incubation at room temperature with twice intermittent inversion followed by centrifugation at 4000 xg for 5 minutes. The supernatant was discarded and 50 µl of CR buffer was added to the pellet followed by addition of 300 µl of the buffer CC and the mixture was vortexed and incubated for 10 minutes at 60°C with inverting the tube every 3 minutes. A volume of 400 µl of the CB buffer was added to the mixture followed by vigorous shaking and centrifugation at 12000 xg for 1 minute. The clear supernatant was transferred to a CC column placed into a 2 ml collection tube followed by centrifugation at 14000 xg for 30 seconds. The flow-through was discarded and the column was replaced into the 2 ml tube. A volume of 400 µl of the buffer W was added into the column followed by centrifugation at 14000 xg for 30 seconds. After washing with W2 buffer, the DNA was eluted using 100 µl of TE buffer.

The isolated DNA was evaluated spectrophotometrically at 260 and 280 nm wavelengths.

Hemi-nested Polymerase chain reaction of BolA DRB3 exon 2

Oligonucleotide primers used for amplification of the exon 2 of BoLA-DRB3 were HL030 (5'-ATCCTCTCTCTGCAGCACATTTCC-3') and HL031 (5'-TTTAATTCGCGCTCACCTCGCCGCT-3'). Both primers were used in the first amplification reaction. Amplification reaction was carried out with 100 ng of DNA (5 µl) in a 25 µl total volume containing 1X PCR buffer; 2.5 mM MgCl 2, dNTPs (100 µM of each), 0.5 µM of each primer and 1 unit of Taq polymerase. The thermal cycling profile for the first amplification was an initial denaturation step of 3 min at 94°C followed by 10 cycles of 25 s at 94°C, 30 s at 60°C, 30 s at 72°C and final extension step of 5 min at 72°C. After first round, a hemi-nested second PCR reaction was carried out with 3 µl of the first-round product into one new tubes containing the same volume and concentration as described above except with primers HL030 and

HL032 (5'-TCGCCGCTGCACAGTGAAACTCTC-3'). The three primers were designed by Van Eijk in a previous study [15].

Primer HL032 is internal to the sequence of the amplified product of the first-round PCR and has eight bases that overlap with primer HL031 (underlined in the text above 5'-TCGCCGCT-3'). The thermal cycling profile for the second round was 25 cycles of 40 s at 94°C and 30 s at 65°C, followed by a final extension step of 5 min at 72°C. Electrophoresis was carried out on 2% agarose gel with 5 µl of PCR product [15].

Restriction fragment length polymorphism (RFLP)

The products of the seminested PCR were subjected in two separate reaction to bot RsaI and HaeIII restriction enzymes [22]. For the RsaI, 10 µl of the PCR products were incubated 5 units of the enzyme for 3 hours at 37°C and at 50°C with 5 units of HaeIII in a total volume of 15 µl for 15 minutes. After digestion, both enzymes were denatured at 65°C for the RsaI enzyme or 85°C for the HaeIII enzyme for 30 minutes., The restriction fragments products were loaded into PAGE containing 12% polyacrylamide and electrophoresed against a 50-bp DNA ladder (GeneRuler^{TM} 50 bp DNA ladder, Thermo Scientific^{TM}, USA). After electrophoresis, the gel was stained with ethidium bromide solution and the restriction fragments were visualized on a UV transilluminator. This was done to identify the pattern of *BolA* DRB3 exon 2. This pattern was demonstrated as the first letter denotes the RsaI patterns and the second letter denotes the HaeIII patterns.

Results

PCR amplification of BoLA class II DRB3 exon 2 alleles of 20 Holstein cows

DNA samples extracted from blood samples of 20 Holstein cows resulted in the amplification of the expected size (284 bp) of the *BoLA* class II DRB3 exon 2 alleles. This was obtained by applying a seminested PCR using specific primers in two PCR runs (Photo 1).

Restriction digestion of BoLA DRB3 exon 2 using RsaI and HaeIII enzymes

As shown in photo (2 a and b), Rsa1 enzyme digestion of the *BoLA* PCR products of 20 cows resulted in 3 profiles, A, B and C. Three profiles were also obtained with the HaeIII restriction enzyme (A, B and C).

Individual BolA DRB3 exon 2 allelic patterns in relation to reproductive traits

As depicted in table 1 (a, b and c), based on the combination between the RFLP patterns produced by the restriction enzymes HaeIII and RsaI on DNA of 20 dairy cows, 5 patterns were seen namely AA, AB, BB, BA and BC (the first letter denotes the RsaI patterns and the second letter denotes the HaeIII patterns). In a similar study performed by Lei in 2012, the 284 bp fragments of the BoLA-DRB3.2 gene were digested with Hae III and 6 RFLP patterns (named AA, BB, CC, AB, AC and BC) were identified [29].

Tracing back for records of productive traits, milk yield and recurrent cystic ovary records were available and tables 1 (a, b and c) show the relationship between the RFLP profiles and the two traits. From the table, it is noticed that the profile AB showed the highest incidence of recurrent cystic ovary, as four animals needed mating for three times for conception. Concerning the milk yield, a cow with the BB profile showed the highest yield (5200 Kg/305 days) followed by those with the BC profile.

Individual BolA DRB3 exon 2 polymorphism in relation to FMD infection and severity

As depicted in table 2 (a and b), cows with the RFLP profiles AA, AB and BB did not attract FMD infection during the endemics that attacked the flock. Meanwhile, two cows with the BC profiles showed severe FMD symptoms. Animals with the rest of profiles showed mild to moderate FMD symptoms according to the farm records.

BolA DRB3 exon 2 allelic profiles in relation to affection with mastitis

Tables 3 (a and b) illustrates the relationship between HaeIII and RsaI RFLP profiles of cows' DNA and the occurrence of mastitis through the milking season. It is noticed that cows with profiles AA, AB and BB were affected with clinical mastitis 3 to 4 times through the production period (305 days). Cows with the profile BC and BA were the least affected with subclinical mastitis.

Discussion

MHC is a polymorphic genetic system that is a cluster of closely coupled genes. It is responsible for the formation of the immune response, macrophage, T-and B-lymphocyte interactions and for the immunological homeostasis support in general. The binding of peptides to MHC molecules triggers acquired immune responses. Therefore, MHC molecule polymorphism forms the diversity of the immune response. In particular, the major histocompatibility complex genes (BoLA) of cattle play an important role in the resistance of the host to diseases [23].

In the current study, all the DNA samples of 20 Holstein dairy cows resulted in the amplification of 284 bp product, the expected size of the targeted amplicon. Digestion with the RsaI enzyme resulted in 3 different profiles A, B and C. Three profiles (A, B and C) were also obtained from the same cows with the HaeIII enzyme. Combination between the

profiles of the two enzymes showed five different alleles namely AA, AB, BB, BA and BC (the first letter denotes the RsaI patterns and the second letter denotes the HAeIII patterns).

In a similar study on 276 Ukrainian red-pied dairy (URPD), 32 BoLA-DRB3 alleles were identified using the two enzymes utilized in this study in addition to the BstyI enzyme [25]. The more the cows' number the more alleles are expected. Only twenty cows were investigated in the current study, as they were the ones with productivity and illness records and two enzymes were applied in this study.

The relationship between HaeIII and RsaI RFLP profiles of cows' DNA and the occurrence of mastitis through the milking season. It was noticed that cows with profiles AA, AB and BB were affected with clinical mastitis 3 to 4 times through the production period (305 days). Cows with the profile BC were the least affected with subclinical mastitis.

In a study carried out by Suprovych and others, the relative risk associated with mastitis in Ukrainian red-pied dairy; it was found that out of 17 BoLA DRB3 alleles, seven were associated with susceptibility and nine with resistance. However, the authors stated that causes of mastitis are different and the condition may be initiated by different factors. The feeding environment, management and genetic constitution are the most leading initiators of mastitis.

The widespread noninfectious mastitis could arise from incorrect milking or poor udder hygiene. Infectious mastitis, on the other hand, is mainly due to invasion of the udder by microbial pathogens. Generally, multiple factors influence the different types of immune responses with certain formation in different bovine populations. In turn, it is unlikely to find determinative BoLA-DRB3.2 alleles that indicate resistance or susceptibility of cows to mastitis [25].

In previous studies, significant associations were reported among BoLA alleles and some infectious diseases of cattle including those prevalent at the early lactation. In a study on Holstein cows, BoLA-DRB3 gene polymorphism was associated with resistance to *S. aureus* mastitis [26].

Furthermore, as cited by Zielak-Steciwko et al. (2024), Yoshida et al. (2009) reported that while certain alleles BoLADRB3 alleles were associated with resistance to mastitis, others revealed an association with an increased incidence of mammary gland inflammation [31, 32].

Also, BoLA allele types were found to be associated with chronic lymphocytosis due to by bovine leukemia virus in Holstein cows where three alleles determined resistance to leukemia while four ones were associated with susceptibility [15,26].

In a study conducted by Lei to investigate the potential association of BoLA-DRB3 gene polymorphism and FMD resistance or susceptibility in Wanbei cattle, the frequency of HaeIII CC and HaeIII BC was higher in healthy cattle than in FMD cattle for the same genotypes. It was therefore, suggested that HaeIII CC and HaeIII BC genotype were actually associated with resistance to FMD. By contrast, HaeIII AA genotype was associated with susceptibility to FMD [29].

In another study conducted in India in 2022, the role of BoLA DRB3 polymorphism was documented in determining susceptibility of vaccinated animals to FMDV infection [30].

Concerning cystic ovary, it was reported by Sharif et al. (1998) that certain BoLA-DRB3.2 alleles were linked to a lower risk of cystic ovarian disease in Holstein dairy cows [27].

Even some indicator traits for innate and acquired immunity were genetically associated with certain BoLA-DRB3.2 alleles in Holstein cows. For example, the IgG2 concentration in was associated with six BoLA-DRB3 alleles in addition to high levels of IgM and complement with lower numbers of mononuclear cell numbers [3,28]. In conclusion, genotype-based selection of dairy cows can enhance the progress of quantitative traits directly and indirectly. Selection can rely on genes directly related to the trait. To date, identifying genes that are associated with significant phenotypic traits has been focused mostly on the major histocompatibility complex (MHC) genes with the DRB gene is being very promising. A kind of association has been detected among traits targeted in the current study and certain BoLA DRB3. 2. alleles.

Conclusion

Despite 20 cows were the all studied animals in this investigation, RFLP analysis has been a valuable tool to document the polymorphism within the BoLA DRB3 exon 2. The allelic profiles based on the RFLP can be used to indicate prospectively productive and/or immunity traits in dairy cows.

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Conflicts of interest

The authors declare that there is no conflicts of interest.

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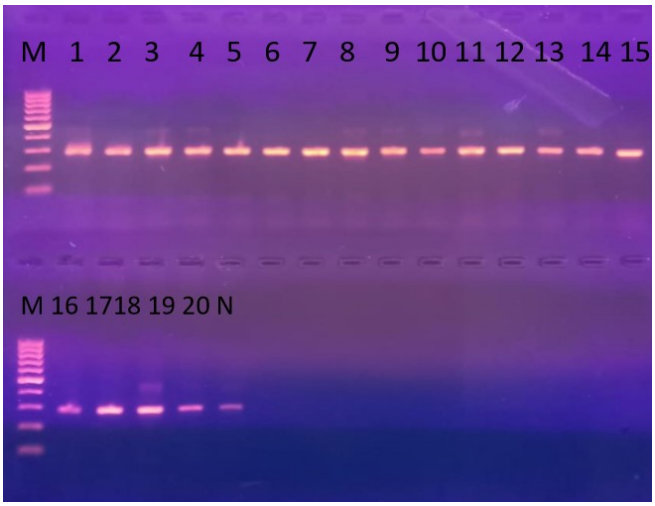


Photo 1: *BoLA* class II DRB3 exon 2 PCR products using seminested PCR. Lane M: 100 bp DNA size marker, Lanes 1 -20: 284 bp PCR products of the *BoLA* class II DRB3 exon 2 of 20 different cows.
Lane N: negative control with no DNA template.

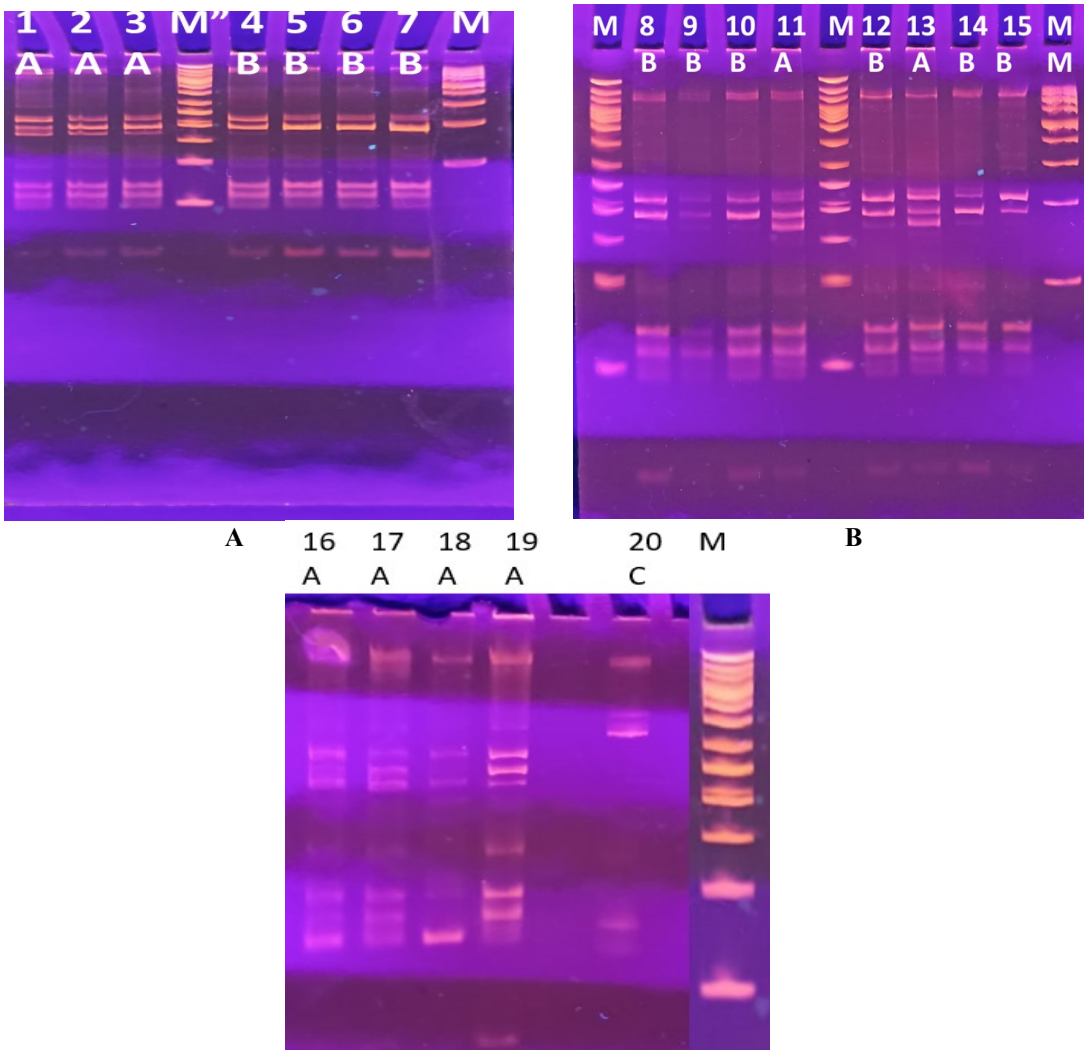
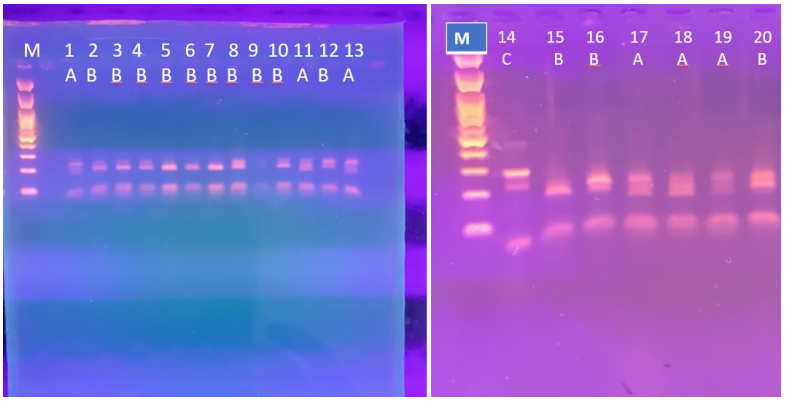


Photo 2: Restriction fragment length polymorphism products of *BoLA* class II DRB3 exon 2 PCR by the Rsa1 restriction enzyme. The digestion products are separated by polyacrylamide gel electrophoresis.
Lane M: DNA size marker, A: lanes 1-7, B: lanes 8-15 and C: lanes 16-20: RFLP digets showing different digestion profiles (A, B and C).



A B

Photo 3: Restriction fragment length polymorphism products of *BoLA* class II DRB3 exon 2 PCR by the Hae III restriction enzyme. The digestion products are separated by polyacrylamide gel electrophoresis.

Lane M: DNA size marker, A: lanes 1-13 and B: lanes 14-20: RFLP digets showing different digestion profiles (A, B and C).

TABLE (1a). Individual BolA allelic patterns in relation to reproductive traits.

Animal number	Pattern	Recurrent cystic ovary	Milk production (Kg X 1000/305 days)
1	A, A	++	3,29
2	A, B	+++	3,6
3	A, B	+++	3,2
4	B, B	+	4,6
5	B, B	+	4,8
6	B, B	+	4,8
7	B, B	+	4.3
8	B, C	++	4,3
9	B, B	+	4.1
10	B, C	+	5,2
11	A, B	++	5
12	B, A	+	5,1
13	A,B	+++	3,5
14	B, C	+	4,9
15	B, B	+	5,1
16	A, B	+++	2,9
17	A, A	++	3.25
18	A, A	+	3
19	A, A	++	3,1
20	C, B	+	4

TABLE (1b). Cystic ovary recurrency in cows in relation to the *BoLA* class II exon 2 RFLP profiles.

Repeats of cystic ovary	RFLP profile					
	AA	AB	BB	BA	BC	CB
One	1		6	1	2	1
Twice	3	1			1	
Three		4				
None						

TABLE (1c). Animal milk production in relation to the BoLA class II exon 2 RFLP profiles.

Milk production in Kg/305 days	RFLP profile					
	AA	AB	BB	BA	BC	CB
~3000	4	3				
~4000			4		1	1
~5000		1	3	1	2	

TABLE (2a). Individual BolA allelic patterns in relation to FMD infection and severity.

Animal number	Pattern	FMD severity
1	A, A	Mild
2	A, B	App healthy
3	A, B	App healthy
4	B, B	App healthy
5	B, B	App healthy
6	B, B	App healthy
7	B, B	Mild
8	B, C	Mild
9	B, B	Mild
10	B, C	Severe
11	A, B	Mild
12	B, A	Moderate
13	A,B	Mild
14	B, C	Severe
15	B, B	App healthy
16	A, B	App healthy
17	A, A	App healthy
18	A, A	App healthy
19	A, A	App healthy
20	C, B	Moderate

TABLE (2b). Foot and mouth disease infection and severity in relation to the *BoLA* class II exon 2 RFLP profiles.

FMD infection and severity	RFLP profile					
	AA	AB	BB	BA	BC	CB
Mild	**1**	**2**	**2**		**1**	
Moderate				**1**		**1**
Severe					**2**	
Apparently healthy	**3**	**3**	**4**			

TABLE (3a). BoLA allelic patterns in relation to affection with mastitis.

Animal number	Pattern		Mastitis degree
1	A, A	3 SC*	4 clinical
2	A, B	3 SC	3 clinical
3	A, B	4 SC	3 clinical
4	B, B	1 SC	2 clinical
5	B, B	2 SC	2 clinical
6	B, B	1 SC	2 Clinical
7	B, B	1SC	2 clinical
8	B, C	2 SC	1 clinical
9	B, B	2SC	2 clinical
10	B, C	2 SC	2 clinical
11	A, B	3 SC	2 clinical
12	B, A	2 SC	1 clinical
13	A,B	4 SC	2 clinical
14	B, C	-1 Sc	2 clinical
15	B, B	2 SC	1 clinical
16	A, B	3 SC	3 clinical
17	A, A	2 SC	5 clinical
18	A, A	3 SC	3 clinical
19	A, A	4 SC	3 clinical
20	C, B	1 sc	2 clinical

*SC: subclinical

TABLE (3b). Mastitis in cows in relation to the *BoLA* class II exon 2 RFLP profiles.

Recurrency of Mastitis/year	RFLP profile					
	AA	AB	BB	BA	BC	CB
7	4	1				
6	1	4				
4		1	2			
3			3	1	2	1
2						

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التفاوت بالقطع الإنزيمي للبديل الوراثي DRB3 exon 2 من BoLA Class II complex وعلاقته بصفات ظاهرية لأبقار هولشتاين حلابة

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الملخص

إن التنوع الكبير في الخارج الثاني (exon 2) من الجزء DRB3 لجينات التوافق النسيجي العظمى القسم الثاني (BoLA MHC class II) يعتبر مؤشر لصفات ظاهرية في الأبقار الحلابة. و عليه فإن هذه الدراسة استهدفت تحديد العلاقة بين بدائل القطع الإنزيمي لمنتج بي سي آر في هذا الجزء الجيني و صفات ظاهرية هامة في الأبقار وهي التحوصل المبيضي، التهاب الضرع، إنتاجية اللبن و الإصابة بفيروس الحمى القلاعية. و تم ذلك باستخدام إنزيمي القطع HaeIII وRsaI على منتج بي سي آر بطول 284 زوج قاعدي. ومن ثم فصل نتائج القطع بطريقة الفصل الكهربي على جل البولي أكريلاميد. أنتج كل إنزيم 3 أنماط قطعية بتطبيق الاختبارات على DNA من 20 بقرة هولشتاين. بالجمع بين الإنزيمين ظهر 5 أنماط لبدائل جينية حيث أظهر النمط AB أعلى معدل إصابة بالتحوصل المبيضي. وقد أظهر النمط BB أعلى إنتجية للبن لموسم الحلب أما الأنماط AA و AB و BB فبم تسجل أي إصابات بفيروس الحمى القلاعية بينما أظهرت بقرتان ذات النمط BC أعراض إصابة شديدة. ارتبطت الأنماط AA و AB و BB بالتهاب الضرع المتكرر بينما كان النمط BC أقلهم إصابة بالتهاب الضرع غير الظاهري. خلاصة القول فإنه بمثابة علاقة بين الأنماط التي تم الحصول عليها من القطع الإنزيمي لبي سي آر للمنطقة BoLA-DRB3 exon 2 و صفات ظاهرية إنتاجية و مناعية في أبقار هولشتاين حلابة. و رغم ذلك فإننا بحاجة إلى دراسات مستفيضة على عدد أكبر من الحيوانات.

الكلمات الدالة:BoLADRB3، القطع الإنزيمي، الفصل الكهربي، جل البولي أكريلاميد، بقر هولشتاين، صفات ظاهرية .